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Impact of roads on forest-living bat species (*Myotis mystacinus* and *Myotis brandtii*) in Sweden

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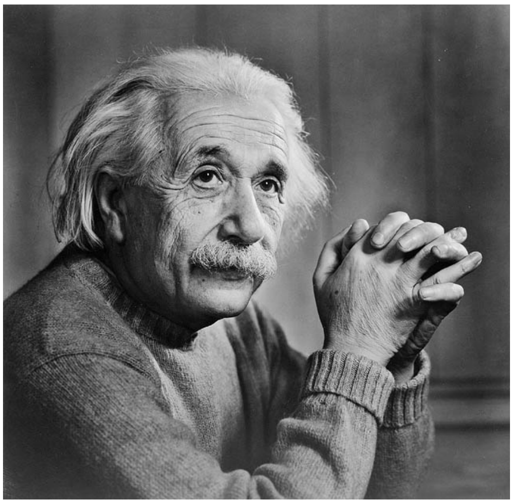

"The world is a dangerous place to live, not because of the people who are evil, but because of the people who don't do anything about it" **– Albert Einstein**

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ABSTRACT

The construction of transport infrastructure represents one of the major ways humans transform landscapes and fragment habitats. Major roads and railways have a wide range of ecological impacts on wildlife. Bat populations appear to be particularly vulnerable due to their high mobility and requirements for widely dispersed resources, which brings them often to road encounters.

In a forest dominated landscape, roads and railways create openings within the landscape, and for species of bats linked to forest habitats, these might act as barriers. Roads and railways also influence bats through noise and light pollution. In some cases, crossing structures for wildlife (both above and under the road) are used to compensate the barrier effect, but their functionality and design are still under evaluation and development. This project is the first research conducted on the impact of roads on bats in a north European hemi-boreal landscape. Landscape and species composition, abundance of bats and the light condition differ from previous studies.

Within all resident bats species in Sweden, *Myotis mystacinus* (whiskered bat) and *Myotis brandtii* (Brandt's bat) are two sibling species that are linked to forest habitats. These species often roost in buildings and forage in forests and wetlands. Road number E18 (between Västeras and Stockholm, Sweden) is a highly used highway. This highway has under- and over passages for wildlife, such as tunnels and wildlife passages. *Myotis* sp. were surveyed along the highway using bat recording stations, and then compared with other open areas and controls. Simultaneously, a study was conducted regarding the flight behaviour of *M. mystacinus* and *M. brandtii* through radio tracking, in the vicinity of the highway and the railway.

The results from the radio tracking showed that *M. mystacinus* and *M. brandtii* were foraging on both sides of the road, selecting forests, streams and hamlet areas. They frequently used the crossing structures, both under and over the road, but in general they did not fly over the road itself. Thus, the road worked as a barrier, and crossing structures were used for foraging and commuting. However, there are exceptions, bats were seen crossing tunnels to reach the vegetated separator of the highway lanes, and foraging along the tree line. Roads themselves are a barrier to *M. mystacinus* and *M. brandtii*, but the adjacent habitats and the area in between the lanes is adequate. Yet special attention should be paid to the dark period of the summer season, when bats showed to be less careful around open areas. Nevertheless, crossing structures are very effective in assuring landscape permeability. By analysing these cases, it is possible to learn more about where and how wildlife passages should be constructed, and to identify problems early in the planning process.

Keywords: *Myotis mystacinus*, *Myotis brandtii*, bat activity, road, barrier, crossing structure.

RESUMO

A construção de infraestruturas de transporte representa uma das maiores ameaças do ser humano à paisagem, causando a sua alteração e fragmentação dos seus habitats. Grandes estradas e linhas férreas impactam na vida selvagem de variadas formas, nomeadamente nas populações de morcegos. Estas populações são particularmente vulneráveis devida a sua grande mobilidade e pelo facto de necessitarem de recursos geograficamente dispersos, o que os traz ao encontro das estradas.

Estas infraestruturas são conhecidas por poderem também trazer benefícios, como possuir uma maior heterogeneidade de vegtação nas suas orlas que pode por consequência ter uma maior disponibilidade de insetos. As orlas podem também formar um abrigo contra o vento e predadores de morcegos. Por último, estas infraestruturas mostraram ser bons marcos para navegação, aumentando assim a permeabilidade entre manchas de habitat.

Numa região onde a floresta é dominante, as estradas e linhas férreas criam espaços abertos na paisagem, e para as espécies de morcegos ligadas a ambientes florestais estes espaços abertos podem atuar como barreiras. Ao intercetarem estes habitats essenciais para estas espécies, há a criação de um obstáculo em rotas já estabelecidas pelos indivíduos. Estradas e linhas férreas influenciam os morcegos também através do volume de tráfego, podendo levar a morte por atropelamento, através poluição luminosa, sonora e química e a própria largura da estrada. Em alguns casos, passagens para fauna são usadas como medida mitigadora, com o intuito de compensar o efeito barreira, mas as suas funções e delineamento estão ainda em avaliação e desenvolvimento. Este projeto é o primeiro estudo sobre o impacto das estadas em morcegos na região do norte da Europa em paisagem semi-boreal. A paisagem, composição de espécies, abundância de morcegos e condições de luz são, por isso, diferentes de estudos anteriores.

Na Suécia ocorrem oito espécies de morcegos, todas insectívoras, das quais *Myotis mystacinus* (morcego-de-bigodes) e *Myotis brandtii*, duas espécies-irmãs que estão ligadas a ambientes florestais. Estas espécies estabelecem frequentemente as suas colónias em edifícios e caçam em zonas de floresta e zonas húmidas. São também conhecidas por utilizar com pouca frequência zonas abertas, sendo por vezes frequentadas por juvenis. A autoestrada número E18, que liga Västerås e Estocolmo na Suécia, é considerada uma estrada com muito movimento. Esta autoestrada tem passagens para fauna, tanto abaixo como acima da estrada, como túneis e ecodutos. Este estudo foi realizado nas proximidades de Enköping entre a última semana de Junho e a primeira semana de Agosto de 2015, como também em Julho de 2016, época em que as fêmeas establecem colónias e se encontram na fase de lactação. Em 2015, a atividade de espécies de *Myotis* foi investigada ao longo da autoestrada através do uso de estações de gravação de ultrassons, e comparada com outras áreas abertas e locais de controlo. A análise deste método foi separada em fase clara e fase escura. Simultaneamente, foi feito um estudo do comportamento de voo das espécies *M. mystacinus* e *M. brandtii* através de rádio-telemetria, método que permite seguir indivíduos que transportam rádio-emissores. Estações de gravação foram também colocadas na área onde foi efetuada a radio-telemetria, na estrada e no ribeiro. Em 2016 foi repetida a medição de atividade das espécies de *Myotis* na estrada, desenvolvida em conjunto com obsevações diretas da estrada e dos túneis que a atravessam. Todas as técnicas foram usadas na estrada, na linha férrea e nas suas proximidades.

Estudos anteriores mostraram que as espécies *M. mystacinus* e *M. brandtii* têm preferência por habitats florestais e ripícolas, e fazerem pouca utilização de zonas abertas, sendo por isso esperado que as espécies se comportassem desta forma. Está também documentado que a abundância e atividade de morcegos deste género diminui com a proximidade da estrada, sendo por isto expectável que a atividade dos morcegos seja menor próxima da estrada quando comparada com a atividade em zonas florestais longe da infraestrutura. Existem poucas observações de indivíduos destas espécies a correr o risco de atravessarem a estrada, sendo assim esperado que a utilização da estrada seja rara ou inexistente. Estas duas espécies foram vistas a utilizar com alguma frequência passagens para fauna, tanto ecodutos como

túneis, no entanto existe documentação de uma maior atividade em túneis quando comparado com a atividade em ecodutos.

Os resultados dos detetores de ultrassons mostrou uma atividade surpreendentemente grande da zona de estrada, maior do que a atividade registada nas clareiras, ao contrário do esperado. Mais tarde, em 2016, este número mostrou ser uma sobreestimação dado que, surpreendentemente, morcegos foram posteriormente observados a atravessar os túneis para alcançar a área entre vias, com o intuito de caçar ao longo deste separador, demonstrando que a atividade registada no ano anterior se devia ao uso de túneis e não ao atravessamento da estrada. O separador possui vegetação e é provavelmente provido de insetos, o que poderá explicar a atividade dos morcegos nesta área. Relativamente às áreas florestadas, a atividade nos ecodutos representa mais do dobro da atividade nas áreas de controlo, o que demonstra uma grande preferência por estas passagens relativamente à floresta conífera circundante. A atividade nas zonas de controlo é inferior à esperada, não sendo suficiente alta para ser considerada significativamente diferente das zonas abertas.

As condições de luz mostraram também ter efeito sobre a atividade de *Myotis* sp.. A sua atividade aumentou no período escuro do verão, quando o número de horas de noite é maior, tanto em zonas abertas como em zonas florestadas. Os morcegos aparentaram estar mais ativos e ser menos precavidos em zonas abertas durante este período, colocando-os em maior perigo. Os ecodutos, pelo contrário, foram altamente selecionados em ambos os períodos do verão, mostrando a sua importância tanto como para movimentação como para alimentação.

Os resultados do seguimento com rádio-emissores mostrou que *M. mystacinus* e *M. brandtii* selecionaram principalmente florestas decíduas e coníferas, ribeiros e áreas rurais, tal como esperado. Estes habitats foram utilizados com frequência nas proximidades da estrada em ambos os lados, ao contrário do que seria expectável. Esta técnica mostrou também que espaços abertos são no geral evitados, de acordo com o esperado. Os morcegos utilizaram frequentemente as passagens para fauna, tanto túneis como ecodutos, e não atravessaram a estrada em si. Isto é, a estrada parece funcionar como uma barreira, mas as passagens para fauna compensam este efeito, permitindo o movimento dentro e entre habitats, mas também como zona de alimentação.

As estradas, em geral, são barreiras para as espécies *M. mystacinus* e *M. brandtii*, dado que estas áreas foram evitadas ao longo do estudo, não implicando que não sejam atravessadas por vezes, mais provavelmente no período escuro do verão. Os habitats adjacentes e o separador que separa as vias da autoestrada mostraram ser adequados. De qualquer das formas, as passagens para fauna foram utilizadas não só como área de alimentação como mostraram ser eficazes em assegurar a permeabilidade da paisagem.

No futuro seria interessante ter um maior conjunto de dados sobre estas espécies, e investigar a razão pela qual alguns indivíduos utilizam o separador entre vias. Seria também interessante investigar o comportamento de outras espécies quando perto de uma auto estrada numa região boreal. Por fim, uma comparação entre o comportamento dos morcegos na presença de passagens para fauna e comportamento dos morcegos na ausência destas, seria de averiguar.

Através da análise da atividade dos morcegos, é possível conhecer os melhores locais onde e como passagens para fauna devem ser construídas e identificar problemas numa fase inicial do processo de planeamento das redes rodoviárias.

Palavras-chave: *Myotis mystacinus*, *Myotis brandtii*, atividade dos morcegos, estrada, barreira, passagem para fauna.

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1. INTRODUCTION

1.1. Infrastructure and wildlife – a worldwide approach

As cities grow, habitats are razed to give way for all kinds of infrastructure. Roads are widespread and increasing, playing an important role for humans by improving communication however, they are also one the most impactful infrastructures on biodiversity, partly due to landscape fragmentation (Evelyn et al., 2004).

Human-caused land-use changes often lead to habitat fragmentation. Habitat fragmentation is defined as the division of a large expanse of natural habitat into progressively smaller patches which finally get isolated from each other. Due to the reduction of integral habitat and gain of edges, the landscape and wildlife can be severely affected (Wilcove et al., 1986; Fahrig, 2003).

Transportation infrastructure is one of the main causes for the changes in abiotic and biotic components within the landscape. Apart from direct destruction for construction, this type of infrastructure has long term effects as reconfiguration of landforms (Coffin, 2007). The richness of several animal and plant groups was found to be negatively correlated to road densities (Findlay & Houlahan, 1997; Coffin, 2007). Road impact is easily underestimated, when the road effects on wildlife are measured a short time after the construction, as Findlay and Bourdages (2000) detected, showing that only after several decades the true road effects are visible when comparing present to historical data.

Roads can have an impact on species and ecosystem dynamics by, for example: increasing mortality rates; decreasing habitat quality; imposing barrier effects, influencing habitat use, dispersal behaviour and genetic structure of populations; different types of pollution (Forman & Alexander, 1998); or even favouring the dispersion of invasive species (Pauchard & Alaback, 2004). On the other hand, it can also have positive aspects, such as: greater vegetation heterogeneity and landscape permeability (Coffin, 2007; Vandevelde et al., 2014). All animal groups appear to be more or less vulnerable to roads, even if due to different motives: birds and mid- and large sized mammals might avoid roads due to traffic disturbance; reptiles and amphibians are attracted or do not avoid roads but are unable to avoid oncoming cars; on the other hand, scavengers can benefit from road-killed animals; and rats and mice are not negatively affected by roads but their predators are (Fahrig & Rytwinski, 2009).

Most of the studies evaluating the impact of infrastructure focus on bird and big mammal populations (Benítez-López et al., 2010; Torres et al., 2016). Bats are also affected by infrastructure but the number of studies are limited compared to other organisms. Bats use different habitats to obtain different resources and are considered good indicators of landscape complexity. Detailed knowledge of their habitat use is necessary for a rational conservation planning on a wide geographic scale (Threffall et al., 2012). This is the first study carried out in Scandinavia about the impact of roads on bat populations focused in the boreal region (Sweden).

1.2. Bats – An overall and *Myotis mystacinus* and *Myotis brandtii* perspective

The order of Chiroptera, which includes more than one thousand species, can be found in almost all terrestrial habitats, with the exception of Polar regions and extreme deserts. All these species are divided into two suborders: Microchiroptera, also known as “echolocating bats”, and Megachiroptera, also known as “flying-foxes”. Bats play important ecological roles: as the primary predators of nocturnal insects they help to reduce agricultural pests; as well as pollinators; and seed dispersers (Kunz et al., 2011). In order to orientate and feed, some species echolocate by emitting rapid sequences of brief high-frequency sound pulses, building up a sonic map of their surroundings (Neuweiler, 1990). Although this ability to create an internal representation of the external world gives bats a great advantage compared to other vertebrates, low fecundity and late maturation increases their extinction risk.

Most echolocating bats are insectivorous and use different habitats to feed, roost and commute, depending on the species. Typically, water bodies, stagnant or running, are strongly selected since they are used as either foraging, due to the high abundance of flying insects, or drinking sites (Vandevelde et al., 2014; Ciechanowski, 2015). Riparian corridors, productive forests, bush lands and productive rural areas are highly selected as well (Threlfall et al., 2012a; Threlfall et al., 2012b). Open arable lands, are less favourable for the majority of species (Threlfall et al., 2012a; Vandevelde et al., 2014; Ciechanowski, 2015). According to bats foraging ecology, it is possible to separate them in five main groups: fast hawking and slow hawking species, foraging in open spaces and forest edges, with large individual home ranges; trawling for aquatic insects; gleaner and hovering species, foraging mostly in between the vegetation, with small individual home ranges; and flycatching and perch-hunting (Norberg & Rayner, 1987).

1.2.1. *Myotis mystacinus* and *Myotis brandtii* – Description, distribution and ecology

Myotis brandtii (Eversmann, 1845) (Fig.1 right) and *Myotis mystacinus* (Kuhl, 1817) (Fig. 1 left) are two sibling species of bats, previously combined in one species. In 1970, Gauckler, Kraus and Hanak described *M. brandtii* as a distinct species, sympatric with *M. mystacinus* even though, they have similar morphology, ecology and behaviour.

Figure 1 – On the left: *Myotis mystacinus* with radio transmitter on the back and reflective ring on the forearm. On the right: *Myotis brandtii*. Photos by Johnny de Jong.

Both species have shaggy fur and its colour can vary from darker- to lighter-brown. The wings, nose and ears are in general blackish-brown (Thompson, 1979). They are small sized bats, their adult weight fluctuates between four and seven grams, even if *M. mystacinus* can be slightly smaller. According to Baagøe (1973), it is possible to distinguish between the two species by the teeth and penis morphology. These bats live in colonies, of up to hundreds of individuals (females during pregnancy and lactation), or solitary (males). Colonies are frequently found in the lofts of buildings, particularly those of timber construction, situated in secluded places or in the vicinity of forests, and rarely found in tree holes (Thompson, 1979; Albayrak, 1991; Boye, 1993).

M. brandtii and *M. mystacinus* occur together throughout most of Central Europe until north of the latitude of 60ºN. In Sweden *M. brandtii* (Fig. 2 right) is found further north compared to *M.*

mystacinus (Fig. 2 left) (Gerell, 1987; Ahlén, 2011). Being typical boreal forest species, these two bats live mostly in or around forests, selecting these habitats together with lowlands with abundant water, e.g. swamp-forests (Lehmann, 1984). Řehák and Beneš (1996) detected greater colonies of *M. brandtii* in large forests. Wetlands of different types are as strongly selected, even if *M. brandtii* appeared to prefer stagnant waters while *M. mystacinus* prefered flowing waters (Taake, 1984). Both species are also encountered in pastures and clearings, especially juveniles that use these areas to forage and practice flying (Adams, 1997).

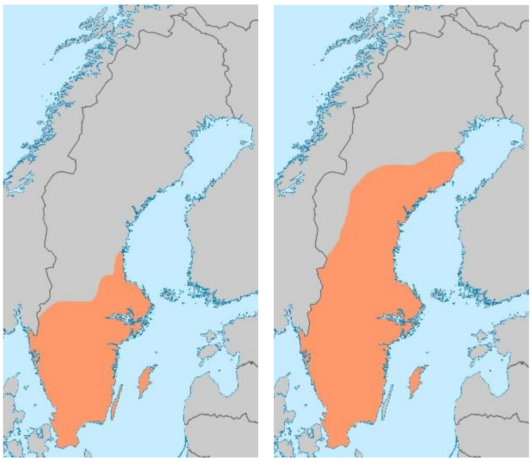

Figure 2 – Distribution of *Myotis mystacinus* (on the left) and *Myotis brandtii* (on the right) in Sweden: dark orange represents the distribution of the species. Adapted from: Ahlén, 2011.

They have large population sizes and wide distributions and are thus, considered to have a status of "least concern" in the IUCN Red List. However, there are threats affecting their survival and distribution, mainly land-use changes, including reduction of habitat, infrastructure development and pesticide use, but also human disturbance to roosts in buildings (Hutson et al., 2008; Coroiu, 2016).

1.3. Impact of linear landscape elements – roads and railways – on bats

When the land cover is mainly divided in a matrix of patches, these are often interconnected by linear landscape elements (Turner, 1989). These elements can be natural, such as hedgerows, waterways and forest edges, or human-made, as roads.

In general, linear landscape elements, might increase landscape connectivity, supporting bats when moving between patches (Kalcounis-Rueppell et al., 2013), and providing heterogeneity, improving foraging habitats (Boughey et al., 2011a; Lesiński et al., 2011). On the other hand, some might cause fragmentation, leading to habitat degradation and decline of bat populations (Hutson et al., 2001). Linear elements benefit bats when tree lines are present (Boughey et al., 2011a) and unbroken (Bennett & Zurcher, 2013), and strengthens when these hedgerows are hard forest edges (Kalcounis-Rueppell et al., 2013) and canopy is high (Abbott et al., 2012b).

Roads, as linear landscape elements, might increase fragmentation of landscapes and populations, which can have substantial impacts, especially when crossing critical habitats (Trombulak & Frissell, 2000). When a road is placed alongside a large patch of natural habitat the degradation is regularly low however, when it crosses smaller habitat patches the degradation tends to be more severe (Forman, 2006). Especially when roads intercept forest patches or rural areas, bat avoidance and mortality are significantly higher (Lesiński, 2007). As a barrier or filter, roads can be avoided because they are a gap in the landscape, mainly when they are within commuting routes (Bennett & Zurcher, 2013). Also traffic volume (Zurcher et al., 2010), chemical (Trombulak & Frissell, 2000), light (Stone et al., 2009) and noise pollution (Siemers & Schaub, 2010) and road width (Fensome & Mathews, 2016) are considered significant factors influencing use of roads by bats. Collisions with vehicles are more frequent than it would be expected for flying animals like bats, particularly for the low flying bats, such as *M. mystacinus* and *M. brandtii* (Lesiński, 2007) However, different species behave differently and are consequently more or less tolerant (Abbott et al., 2012a; Abbott et al., 2012b; Berthinussen & Altringham, 2012b; Threlfall et al., 2012a; Ciechanowski, 2015).

Besides the negative effect, road and railway edges can provide: suitable foraging habitat; linear landmarks for navigation; and shelter to wind and predation (Limpens & Kapteyn, 1991). Probably due to these reasons, Limpens et al. (2005) and Kalcounis-Ruppell et al. (2013) found that bat flight patterns were consistently parallel with the road. Moreover, Valdevelde et al. (2014) found that bat activity in railway edges was similar to nearly all surrounding habitats, except for aquatic habitats.

Even if these structures occupy only a small percentage of landscapes, the ecological effects can be significant over a much larger area (Ward et al., 2015), and in a forest dominated landscape, the negative impact of roads as barriers might be particularly severe. Kerth & Melber (2009) found out that *M. bechsteinii,* that hunted close to a road, had smaller foraging areas, compared to the ones hunting far from the road. Zurcher et al. (2010) discovered that bat road avoidance is proportional to traffic volume. Later, Berthinussen & Altringham (2012a) detected a decrease in bat activity and diversity near roads, as well as an absence of bats flying over the roads, suggesting that these structures may have caused the abandonment of previous flight lines. Additionally, Kitzes & Merenlender (2014) demonstrated that, along the first hundred meters from the roadside, bat activity decreased with the proximity to the highway. Regarding mortality, Medinas et al. (2013) study revealed that road-kills occurred mainly in road segments with higher traffic volume and that crossed high-quality habitats. However, Abbott et al. (2012b)'s results show evidence that highways are not absolute barriers to the movements of bats if crossing structures, as tree-lines and river bridges, assure landscape permeability.

The impacts of railways are not as studied as those of roads, but probably their impact is smaller. Railways networks are not as vast, and often have smaller width and weaker traffic, especially when bats are active (Vandevelde et al., 2014).

The highways' impact depends greatly on the surrounding landscape and its mitigation measures. Because bat populations can decline or relocate, depending on their sensitivity (Berthinussen & Altringham, 2012a), it is very important to consider the connectivity first between the different patches (Threlfall et al., 2012a), and then between the overall landscape, when including the road and railway networks (Vandevelde et al., 2014).

1.3.1. Mitigation measures: crossing structures – over- and underpasses

Crossing structures, such as over- and underpasses, carrying streams and forests, result in an increase of landscape permeability (Ward et al., 2015) and appear to play an important role in bat movements (Kerth & Melber, 2009; Abbott et al., 2012b; Berthinussen & Altringham, 2012a). They are used by bats for both foraging and commuting (Abbott et al., 2012a). Wildlife overpasses are bridge-like structures with variable size designed for use by fauna, covered by grass, shrubs or trees (Corlatti et al., 2009). Underpasses are tunnels or passes under bridges, carrying low vegetation or rivers that provide passage to wildlife (Glista et al., 2009). According to Abbott et al. (2012a, 2012b) and Ward et al. (2015), both types of structures are used by bats as crossing routes, even if underpasses appear to be preferred, those with rivers or streams. *M. mystacinus* and *M. brandtii* were found to frequently use also tunnels of different sizes, but rarely bridges (Bach et al., 2004). Even though, the presence of these structures per se does not assure the bats safety, their location should coincide with their commuting routes, otherwise they are ineffective for bats (Berthinussen & Altringham, 2012b). Although bat abundance in and around these structures is higher, Medinas et al. (2013) detected a higher mortality due to collision with vehicles as well. Overall, when passages are available, these are preferred to over-road routes.

The effect of roads, its edges and crossings structures on bats is probably dependent on the surrounding landscape, as well as on bats' foraging ecology and wing morphology (Abbott et al., 2012a; Kerth & Melber, 2009; Vandevelde et al., 2014; Ward et al., 2015). Forest-linked species, such as some *Myotis* species, have shown a greater road avoidance (Berthinussen & Altringham, 2012a), to be impacted negatively by edges (Vandevelde et al., 2014) and to use more crossing structures in order to commute, than aerial hawking species (Abbott et al., 2012b). Though, it is documented that this species sometimes fly over roads (Abbott et al., 2012b; Berthinussen & Altringham, 2012b; Ciechanowski, 2015).

Mitigation measures are applied to lower the barrier effect and its impact. Crossing structures can facilitate safe crossing of roads and railways however, their success in promoting movements remains unclear for bats. *M. mystacinus* and *M. brandtii*, as forest-living species, are potentially more vulnerable to mortality and fragmentation by roads, when compared to aerial hawking species, due to its foraging ecology (Kerth & Melber, 2009; Zurcher et al., 2010; Berthinussen & Altringham, 2012a and 2012b; Abbott et al., 2012a; Vandevelde et al., 2014).

1.4. Objectives

This study intends to answer the following questions:

I. How are forest-living species of bats, *M. mystacinus* and *M. brandtii*, affected by roads in a forested dominated landscape in the boreal region?

In order to answer this question, habitat selection, foraging behaviour, and avoidance response to open spaces during the light and dark period of the summer season, were investigated. It was shown in previous studies that bats, including forest-living species, are negatively affected by road infrastructure. It is expected that *M. mystacinus* and *M. brandtii* avoid roads and open spaces, and that bat activity is relatively higher during the dark period of the summer season.

II. How will crossing structures impact *M. mystacinus* and *M. brandtii* behaviour?

To answer this question, bat behaviour while using these structures was observed and their importance in bats movements was evaluated. Previous studies show that bats, including forest-living species, use and benefit from crossing structures, mostly from underpasses. It is expected that *M. mystacinus* and *M. brandtii* use the structures for both commuting and foraging.

The boreal region differs in landscape and species composition, abundance of bats and their behaviour and also light condition. All these features differ from previous studies.

2. MATERIAL AND METHODS

Eight species belonging to the *Myotis* genus were observed in Sweden: *Myotis alcathoe*; *M. bechsteinii*; *M. brandtii*; *M. dasycneme*; *M. daubentonii*; *M. myotis*; *M. mystacinus*; and *M. nattereri*. To date, 2 460 kilometres of highways (Trafikverket, 2014) have been constructed, and the number is rising.

This study is divided into two parts with different methodology. The first part is based on acoustic surveys and aims on investigating activity of *Myotis* sp. along a highway. This part allowed to evaluate habitat selection for both light and dark period of the summer season and avoidance of open spaces, in order to answer question I. In addition, bat activity at the wildlife passage was required to answer question II. The second part is based on both radio tracking and surveys, and the purpose is to investigate behaviour of *Myotis mystacinus* and *Myotis brandtii* near a highway. Habitat selection and foraging behaviour of these species was essential to answer question I, the usage of the wildlife passage and tunnels allowed to answer question II.

By combining methods, the risk of under- or overestimation is minimized. Acoustic surveys can sample wide areas and multiple geographic locations at low costs and gather large numbers of recordings belonging to several species. On the other hand, radio tracking allows the gathering of information attributed to particular individuals, which means gender, age, weight and other characteristics are known, and also there is no risk of overestimation.

2.1. Surveys along the road

This part of the study took place around Enköping, a city located in south-central Sweden (N59º38’, E17º4’), along the highway that connects Oslo and Stockholm, road number E18. The landscape in the study area is dominated by coniferous forest, but deciduous and mixed forest are also common. There are also open areas, mostly fields, whereas lakes and large streams are absent.

2.1.1. Placement of the ultrasound recorders

In order to compare bat abundance in the road with other open areas, ultrasound recorders (Fig. 3) were placed by the road, in gaps in the forest and at control sites. Ultrasound recorders were also placed at two different wildlife passages. Bat activity was recorded with Pettersson D-500X (Pettersson Elektronik AB) ultrasound recorders (Fig. 3). Bat calls were recorded from 22:00 until 04:00 each night, with the following settings: recording sensitivity (very high), sample frequency (500kHz), pretrigger (off), rec-length (3), HP-filter (y), auto rec (y), input gain (60), trigger level (30) and interval (5).

Figure 3 – Ultrasound recorders: device on the left; covered and ready to hang up on a tree, on the right. Photos by Rita Luz.

Field work was conducted between June 23rd and August 7th 2015. All sampling sites (34) were repeated seven times, once a week for seven weeks (a total of 238 samples).

A. Major road

E18 is a double lane highway (Fig. 4 left) with a separator with trees, shrubs, stones, etc. (Fig.4 right). The traffic volume varies greatly depending on the period of the year and the time of the day. On average, from 2011 to 2014 there are 108 998 vehicles per year (Tafikverket, 2014).

Figure 4 – E18: right lane and separator, on the left; separator that divides the lanes on the right. Photos by Johnny de Jong.

Ultrasound recorders were placed at eight sites by the road, in the trees located in the separator between both lanes, in order to record the bats that crossed the road. The sites were located around 1-3km away from each other, between Enköping and Ekolsund. Along this road section, 80% of the edges are covered by forest.

B. Gaps between forests

With the purpose of testing if bats avoid open areas in general, gaps between forest patches around Enköping were selected (Fig. 5). Suitable sampling sites were searched within the landscape along the road (up to 5km each side). The selected gaps needed to meet two criteria: the same width as the road (40 to 80m); and a place in the middle of the gap (e.g. a tree) where the ultrasound detector could be positioned. Eight suitable gaps were considered.

Figure 5 – Gaps: the ultrasound recorders were placed in the trees in the middle of the gaps. Photos by Johnny de Jong.

C. Control

Control sites were located in the forest near to the highway and the gaps (Fig. 6), in order to relate the abundance in open areas with the general abundance in the area. All control sites were placed within a distance of 100 to 1000m from gap or road sites, in semi-open mature forest (coniferous, deciduous or mixed) but never near the edge. In total, ten control sites were used.

Figure 6 – Forests: the ultrasound recorders were placed in the trees within the forest. Photos by Rita Luz.

D. Wildlife passages

Bridges with a roof of natural vegetation are used as wildlife passages over E18. In this study, two of these bridges were included.

The first passage (Passage A) (Fig. 7 left) has dense coniferous forest on the roof. West of the passage there is a small stream surrounded by natural vegetation. The second passage (Passage B) (Fig. 7 right) has more open forest. The east side is surrounded by sparse coniferous forests and the west side by farmlands. At passage A, a small river flows on the west side, crossing the road under it. Both passages have natural vegetation and are 100m wide.

Figure 7 – Wildlife Passages: on the left Passage A (Photo by Johnny de Jong); on the right Passage B (Photo by Rita Luz).

Four ultrasound detectors were placed at each passage, one in the middle of the passage (within the forest), one in the edge of the forest and two in forest adjacent to the passage.

2.1.2 Data analysis

After recording, the data was analysed using Omnibat (Ecocom AB, Sweden) in which bat sounds were automatically sorted by species and separated from noise sounds. However, all sounds were also checked manually, and Pettersson Elektronik AB was used as a complementary analysis programme. Each bat pass was considered as one observation. Though, if there was more than one species in the same recording, the number of observations was equivalent to the number of species in that recording. Bat passes belonging to *Myotis* sp. were the only considered and were not separated in species.

0 1,25 2,5 5 7,5 10 Kilometer

Figure 8 – Study area: Enköping city in the middle and road E18 in dark red. Circles represent the sites where ultrasound recorders were placed: (grey) road sites 1 to 8; (orange) gap sites 1 to 8; (green) control sites 1 to 10; (blue) wildlife passage sites 1 to 8. Closer view in supplementary material.

Comparisons of medians between the activity at road sites, gaps between forests, controls and wildlife passages during the whole summer season, were made using a Kruskal-Wallis test. Moreover, a comparison of medians of the activity at the different habitats between the light and dark period of the summer season (light period starting on the June 23rd until July 11th 2015 and dark period starting on the July 20th until August 7th 2015), was performed by a Mann-Whitney U test. Finally, comparisons of medians between the activity at all the habitats within the light period and within the dark period of the summer season, were performed by a Kruskal-Wallis test. Significance level was set to 95% (α=0.05).

2.1.3. Direct observations along the road

Based on the results of the ultrasound detectors along the road, direct observations were conducted in the summer season of 2016. The objective was to map the flight paths of *Myotis* bats where the number of bat passes recorded by the road were higher than 10 observations: Road03, Road06, Road07 and Road08 (Table S1). Three of these points were selected: Road03, Road06 and Road07 (Fig. 9). At two of these sites (Road03 and Road07) there was an underpass nearby (around 150m) (Fig. 10). The third site (Road06) was located 600m away from the nearest underpass. Two observers were located at strategic positions from where the road and the underpass were visible. Ultrasound detectors and head-torches, even if the light condition at this time of the summer were good enough without any artificial light, were used to find the bats.

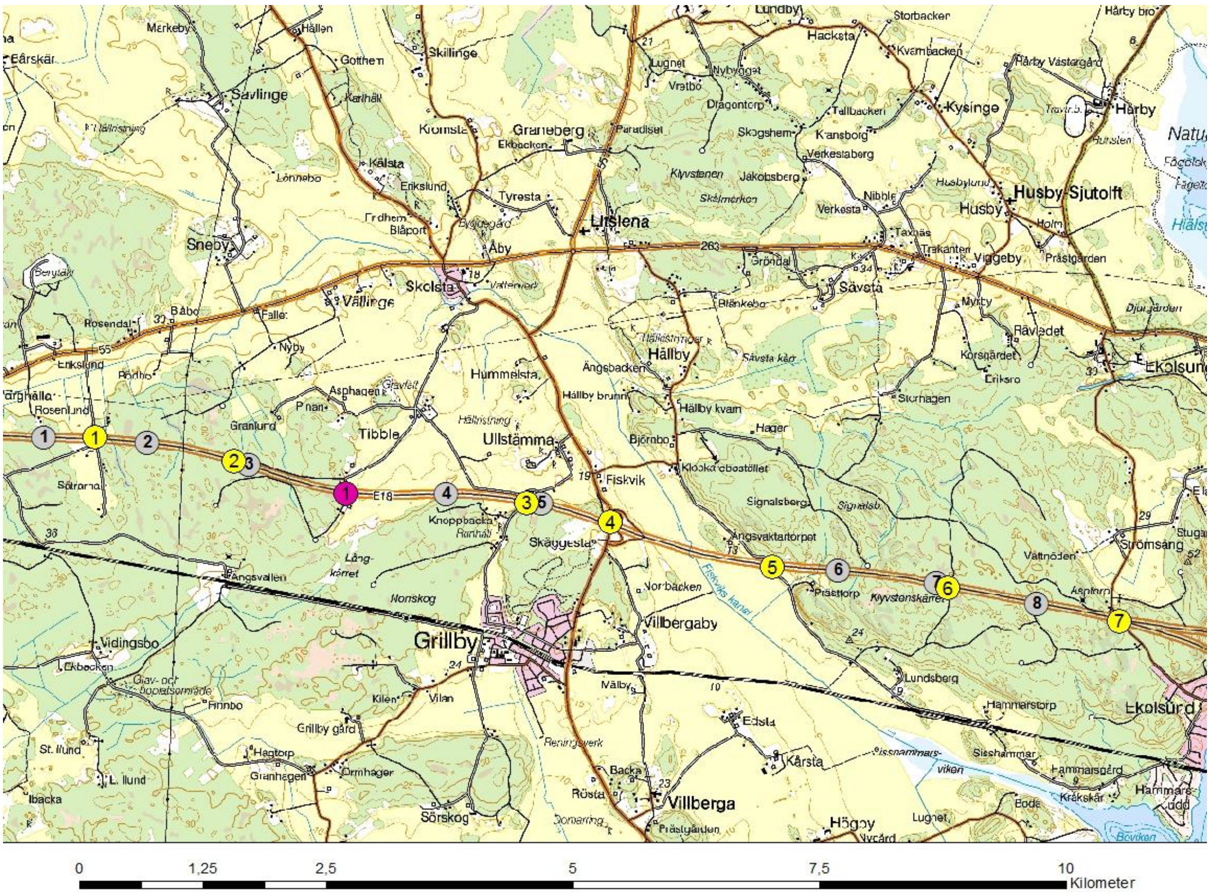

Figure 9 – E18: (grey) road sites 1 to 8; (yellow) tunnels crossing the road 1 to 7; (purple) bridge crossing the road.

Figure 10 – Underpasses: tunnel number 2 near to sampling site Road03 (on the left); tunnel number 6 near to sampling site Road07 (on the right).

Simultaneously, two ultrasound recorders per night were placed at the sampling sites from the previous year. The sampling in all sites (3) were repeated three times (a total of 9 samples).

The fieldwork was carried out ten nights from the July 11th to the 20th 2016.

The ultrasound recorders' settings and bat sound analysis were identical to part 2.1.2. (above).

2.2. Habitat use of *Myotis mystacinus* and *Myotis brandtii*

This part of the study was conducted in the area around Ullbro (N59º39', E17º0') and Ullunda (N59º38', E17º1'), 5 km west from the city of Enköping. The area is dominated by agriculture and coniferous forest (Fig. 12). A railway (Fig. 11 left) and a highway E18 (Fig. 4 left) have been constructed parallel to each other through the area and divide it into two parts. Under the road and the railway crosses a tunnel (Fig. 11 right) with running water and vegetation, and above the road and the railway there is a wildlife passage (bridge) (Fig. 7 left).

Figure 11 – On the left: railway parallel to E18. On the right: tunnel under the road with vegetation and a stream. Photos by Johnny de Jong.

2.2.1 Radio tracking

Two *Myotis* sp. colonies were included in this part of the study. A *M. mystacinus* colony (Fig. 13 left) was located 250 m north of the railway and a *M. brandtii* colony (Fig. 13 right) was 350 m south of the highway. The followed bats were using different habitats within 1.3km from the colonies and, therefore a landscape with radius of 1.5km from each colony was considered in the analysis (Fig. 12).

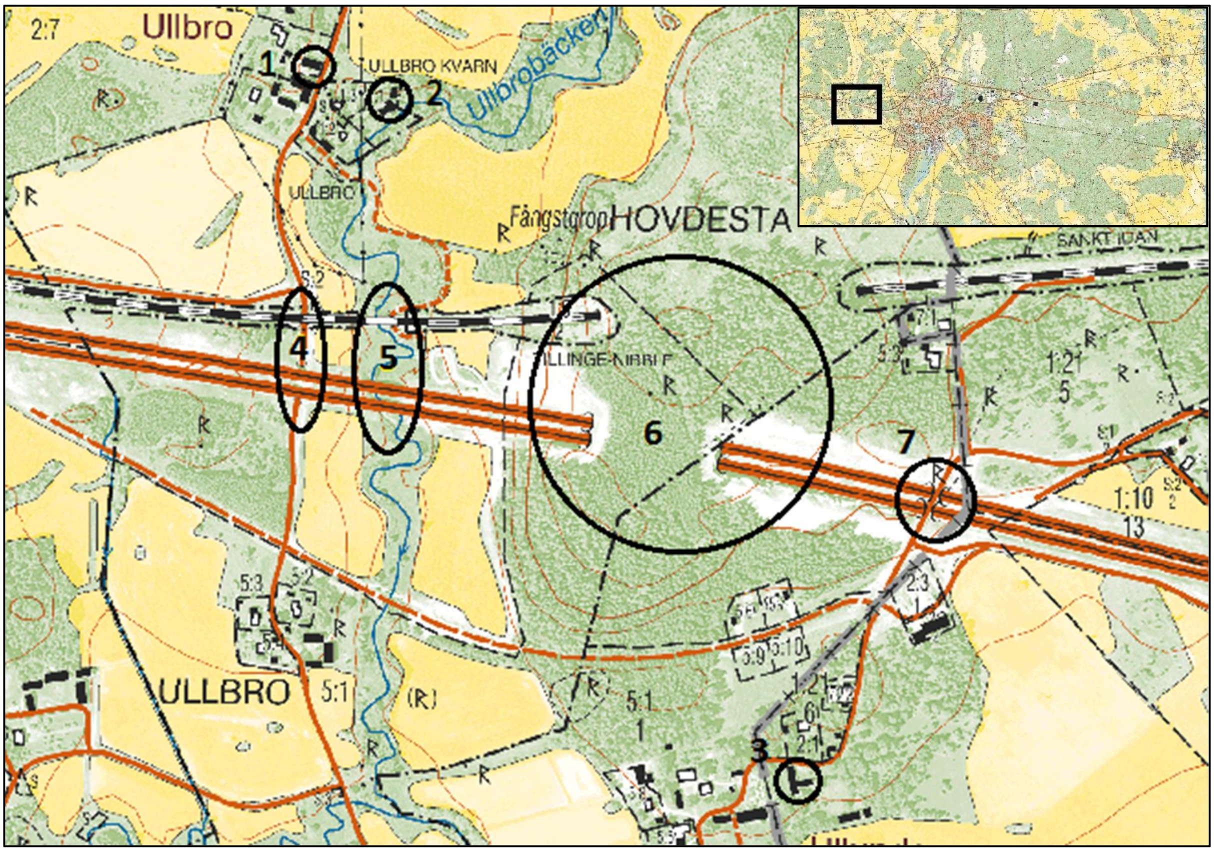

Figure 12 – Area of Ullbro: (1 and 2) *M. mystacinus* colony; (3) *M. brandtii* colony; (4) car tunnel crossing both the highway and the railway; (5) tunnel under the road and the railway with vegetation and a stream; (6) wildlife passage (overpass); (7) car bridge.

Fieldwork started on June 26th and lasted until August 1st 2015, during late pregnancy and lactation of the bats. Tracking lasted from around 21:45 until the bat returned to the colony and remained resting there for some time.

Figure 13 – Barns where *M. mystacinus* (on the left) and *M. brandtii* (on the right) established their colonies. Photos by Rita Luz.

Bats were caught with mist nets outside of the colonies or at their known hunting grounds. After capture, bats were gently removed from the net and identified to species and gender. Only adult individuals were used. Rings with reflective tape were put on the forearms for visual identification (Fig. 1 left). A radio transmitter (Holohil systems Ltd) was then glued (Sikema AB) to the between the bat's shoulder blades (Fig. 1 left). The bats were then released to adapt to the transmitters for at least one night.

Bats were located with two different sets of radio receivers and external antennas (FollowIt RX 98). One was used at different listening points. These points were reached by foot or by car and the direction, in degrees, where the bat was heard was estimated through the intensity of the signal. In addition, the distance from the bat to the receiver was also estimated (visual, close, middle, middle far or far), as its activity (resting or flying). The bat location was registered every five minutes until it flew away. The other set of equipment was used to get closer to the bats, in order to map the flight path, and to find where it forages. When close to the bat, the individual was identified with the help of the reflective tape placed on the forearm.

In total, 15 bats were tracked, 11 *M. mystacinus* and four *M. brandtii* throughout 37 nights (in five nights the rain was too heavy to track) (Table S2). Each night, one or two bats were tracked and each bat was tracked during one to four nights.

2.2.2. Placement of the ultrasound recorders

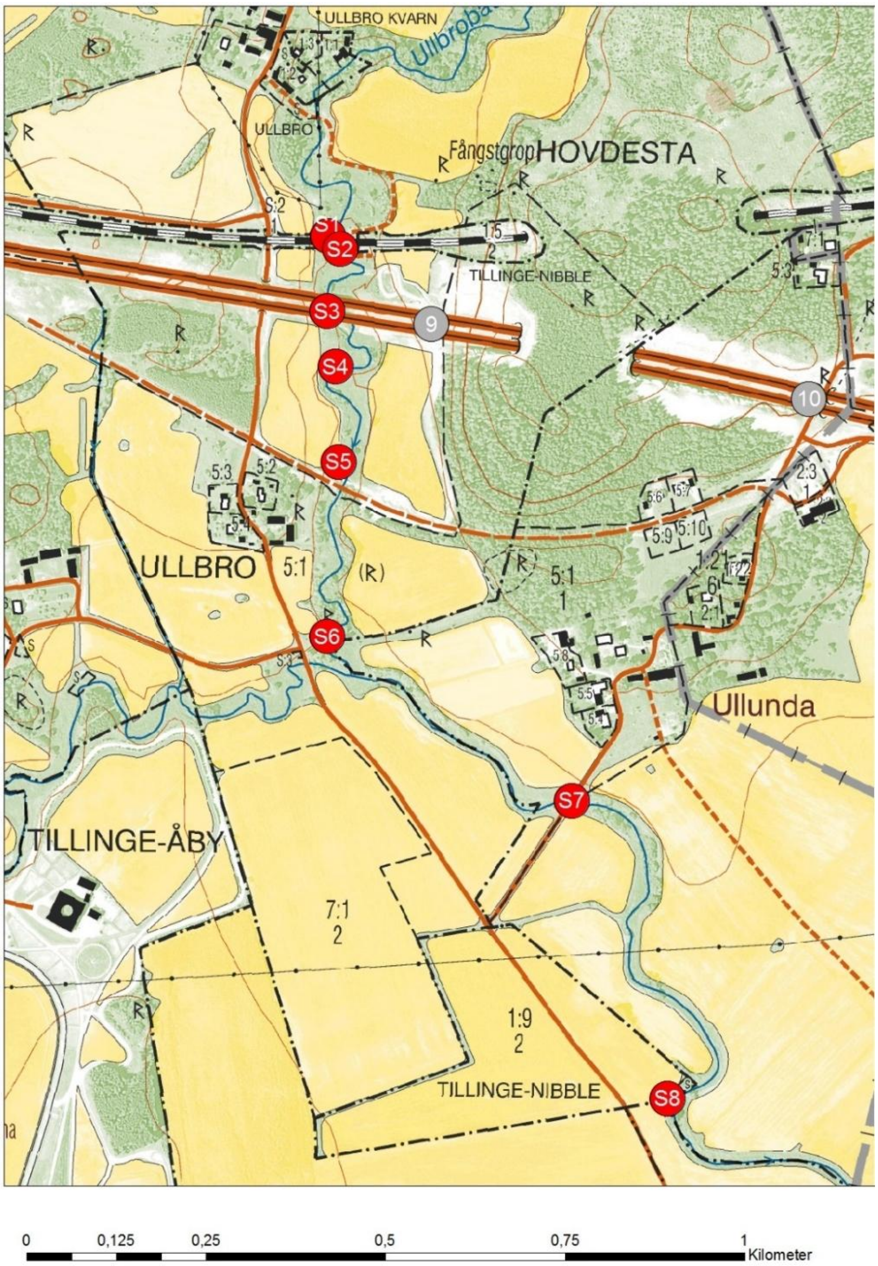

Figure 14 – Ultrasound recorders from Ullbro: (grey) road sites 9 and 10; (red) stream sites 1 to 8.

To support radio tracking and get more data on the habitat use close to the colonies, ultrasound recorders were placed in the area around the colonies, by the road and the stream (Fig. 14). Two ultrasound detectors were placed by the highway, E18, one on the road and one on the top of a car bridge, pointing down to the motorway. Eight more ultrasound recorders were placed along the stream, leading from the railway towards south.

Ten sites were considered and each site was sampled twice (a total of 20 samples) between June 26th and August 1st 2015.

2.2.3. Data analysis

Concerning radio tracking, since the followed bats left the colony, each five minutes the bat's position was attributed, which was counted as one observation. All observations of *M. mystacinus* were added together, as well as observations of *M. brandtii*, creating two separated analyses with each species and their habitat availability. Positions were after associated to the habitat they were inserted in, using geographic information systems (ArcMap and QGIS).

The number of observations in each particular habitat was compared with the proportion of that habitat which means, its availability, via χ^2 test. Selection or avoidance of habitat categories was detected by constructing individual confidence intervals for each habitat using Z statistic. Confidence limits were set to 95% (α=0.05, but with Bonferroni correction α=0.006). Each confidence limit was compared with the expected proportion based on the availability of the habitat. If the expected value was within the confidence interval, then it was concluded that the habitat was used in proportion to its availability. If the expected value was lower, the habitat was selected, while if higher, then the habitat was avoided (Neu et al., 1974).

Special attention was paid to open areas, to investigate if bats avoid open areas in general or specifically the road. Also, it was important to determine if bats forage close to the road and how they behave close to it.

The ultrasound recorders' settings and bat sound analysis were identical to part 2.1.2. (above).

3. RESULTS

3.1 Surveys along the road

Within the eight road sites, eight gaps in the forest, ten control sites and eight wildlife passage sites, in total, 9696 bat passes were recorded of five taxa (*Myotis* sp., *Eptesicus nilssonii*, *Nyctalus noctula*, *Pipistrellus pygmaeus* and *Plecotus auritus*). Though, this study is focused only on *Myotis* genus, with 3751 bat passes (Table 1).

Table 1 – Number of bat passes of *Myotis* sp. and correspondent percentage for each habitat class.

Sites	Number of bat passes	Percentage (%)
Road	834	22.2
Gap	359	9.6
Control	852	22.7
Wildlife Passage	1706	45.5
Total	3751	100.0

The distribution of *Myotis* sp. passes among habitat classes was strongly uneven. As represented in the Table 1, among 3751 *Myotis* sp. observations, 1706 were recorded in the wildlife passages (representing 45.5% of all *Myotis* sp. observations), 852 in control sites, 834 by the road and 359 in gap sites.

These results revealed that there was much higher activity in wildlife passages, while the road and gap sites together had 30% of all *Myotis* sp. passes.

3.1.1. Activity of *Myotis* sp. during the whole summer season

The Kruskal-Wallis test revealed that activity of *Myotis* sp. appears to differ between habitat classes (H=32.695, p-value<0.001) (Fig. 15). Post-hoc tests revealed that concerning road- and gap sites (p-value=0.960), the difference appears to be slight and far from significance. The difference between road- and control sites (p-value=0.068) was also not significant, but close to the significance level. Regarding gap- and control sites (p-value=0.138), the difference was not significant. And from the p-value between wildlife passages and the three remaining classes (road: p-value<0.001; gap: p-value<0.001; control: p-value=0.005), the difference between medians was significant.

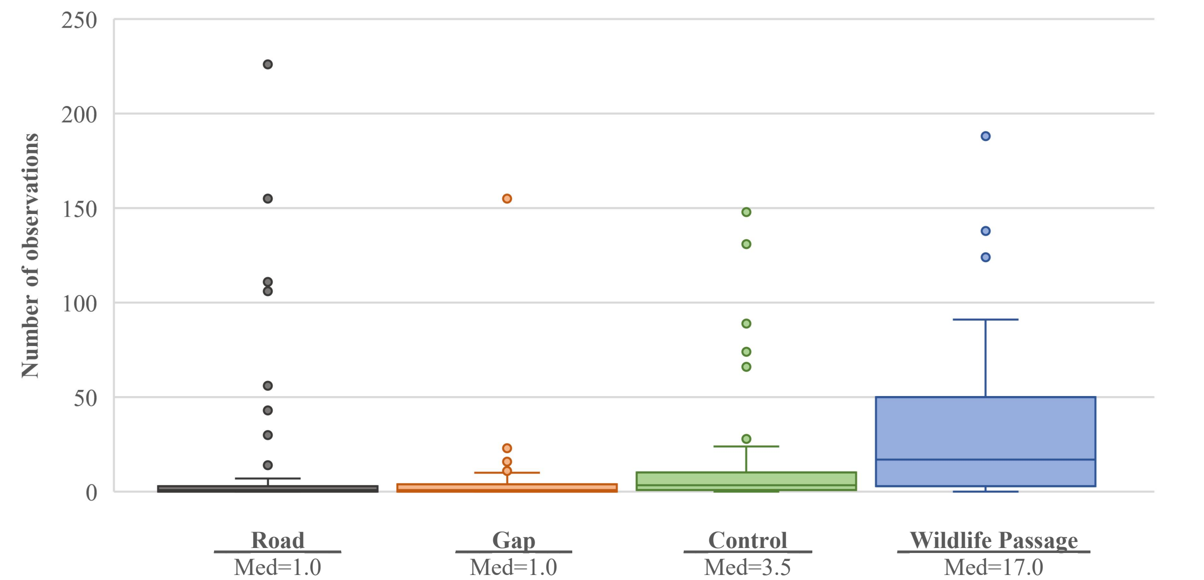

Figure 15 – Box plot representing the number of observations of *Myotis* sp. per habitat class within the whole summer season. The box represents the middle 50% of the observations, from the lower to the upper quartile. The mid-point of the box is the median. The whiskers represent the observations outside the middle 50%. The dots represent outliers. The median (Med) for each class is shown in the x axis.

3.1.2. Comparison between activity of *Myotis* sp. during the light and dark period of the summer season

The activity of *Myotis* sp. (Fig. 16) appears to vary along the summer season, according to the Mann-Whitney test. By the road (W=477.5, p-value=0.018), there was significant difference between medians in the light and dark periods. In gap- (W=423.0, p-value=0.001) and control (W=674.5, p-value<0.001) sites the difference was significant, with very low p-values. On the other hand, in the wildlife passage (W=554.5, p-value=0.495), it is possible to assume that there was no difference between the two periods.

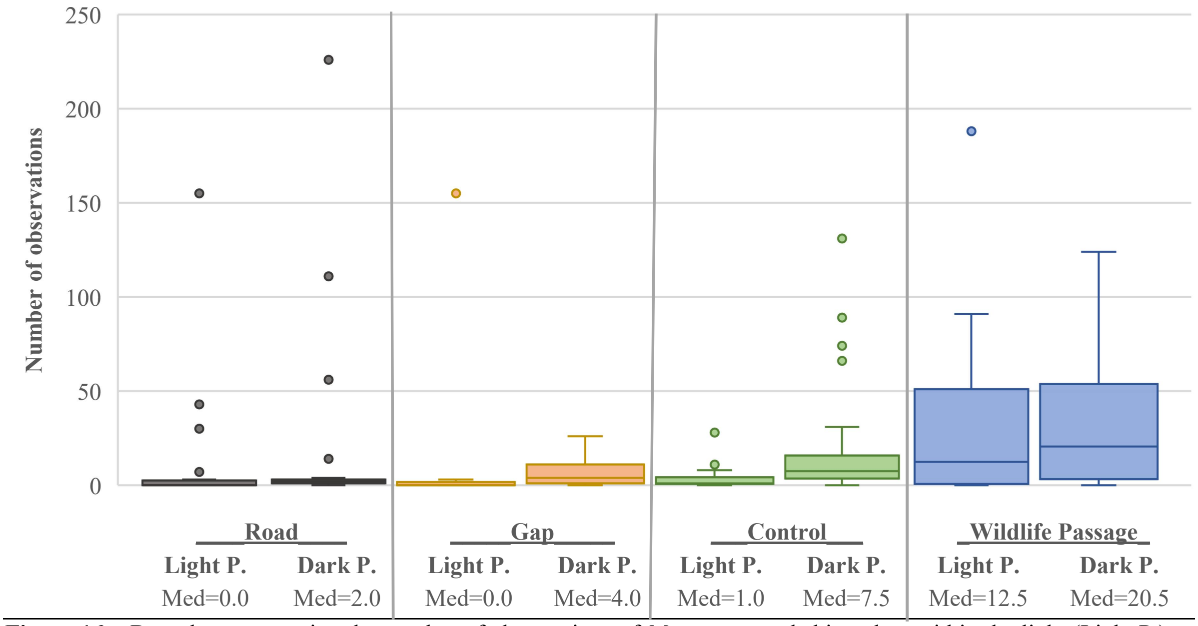

Figure 16 – Box plot representing the number of observations of *Myotis* sp. per habitat class within the light (Light P.) and dark (Dark P.) periods of the summer season. The box represents the middle 50% of the observations, from the lower to the upper quartile. The mid-point of the box is the median. The whiskers represent the observations outside the middle 50%. The dots represent outliers. The median (Med) for each class is shown in the x axis.

3.1.3. Activity of *Myotis* sp. during the light period of the summer season

A Kruskal-Wallis test revealed that activity of *Myotis* sp. between habitat classes (Fig. 16) differed during the light period of the summer season (H=18.811, p-value<0.001). With post-hoc tests, it is possible to observe that, during the light period of the season, road- and gap sites (p-value=0.999), road- and control sites (p-value=0.212) and control- and wildlife passages sites (p-value=0.094) were not significantly different. Gap- and control sites (p-value=0.088) were also not considered significantly different, though the p-value is near to the significance level. The remaining comparisons appear to be significant: road- and wildlife passages sites (p-value=0.023) and gap- and wildlife passages sites (p-value=0.011).

3.1.4. Activity of *Myotis* sp. during the dark period of the summer season

During the dark period of the summer season, activity of *Myotis* sp. between habitat classes (Fig. 16) was considered significantly different (H=13.195, p-value= 0.004), though all the previous differences become less marked. Post-hoc tests showed that road- and gap sites (p-value=0.625), road- and control sites (p-value=0.063), gap- and control sites (p-value=0.408) and control- and wildlife passage sites (p-value=0.743) were not significantly different. The difference that existed in the light period becomes less marked in the dark period in road- and wildlife passage sites (p-value=0.077) and in gap- and wildlife passage sites (p-value=0.081). Noting that the comparison between road- and control sites, road- and wildlife passage sites, and gap- and wildlife passage sites, are close to the significance level.

Besides, it is noticeable that the medians were generally higher on the dark period of the season when compared with the light period.

3.1.5 Direct observations along the road

In total, 38 hours were spent observing the road and the underpass and 128 bat passes were recorded by the ultrasound recorders (Table 2).

Table 2 – Mean, standard deviation, minimum and maximum of the number of *Myotis* sp. passes recorded by the ultrasound recorders placed by the roads (Road03, Road05 and Road07).

Mean	12
Standard deviation	15
Minimum	0
Maximum	41
Total	128

No bats were observed crossing the road but they were frequently observed using the underpass. Moreover, bats were seen flying along the separator.

3.2. Habitat use of *Myotis mystacinus* and *Myotis brandtii*

3.2.1. Radio tracking

The landscape surrounding both *M. mystacinus* and *M. brandtii* was divided into the following biotopes: hamlet, coniferous forest, deciduous forest, *Salix* plantation, stream, open grassland, field and road (Table 3, Fig. 17 and 18).

Table 3 – Description of the considered biotopes and their availability in hectares (ha) and percentage (%) for *M. mystacinus* and *M. brandtii*.

		Habitat availability for *M. mystacinus*		**Habitat availability for *M. brandtii***	
Biotope	**Description**	**(ha)**	**(%)**	**(ha)**	**(%)**
Hamlet	Area with some buildings, such as houses and barns and small gardens. If the property had a big lawn and no buildings, the open area was considered as open grassland.	26.8	3.8	38.2	5.4
Coniferous forest	Dominated (>70%) by coniferous trees.	315.7	44.5	164.7	23.2
Deciduous forest	Dominated (>70%) by deciduous trees.	12.5	1.8	12.0	1.7
Salix plantation	Managed forest containing only *Salix sp.* trees (for biofuel purpose).	35.8	4.6	32.3	4.6
Stream	Area with running water surrounded by natural riparian vegetation (trees, bushes and grassland).	21.5	3.0	25.9	3.7
Open grassland	Open grasslands used as pasture or without any specific use.	38.3	5.4	27.8	3.9
Field	Open land used for agriculture.	205.5	29.0	349.9	49.3
Road	Area containing the highway and railway and the space in between.	53.3	7.5	59.0	8.3

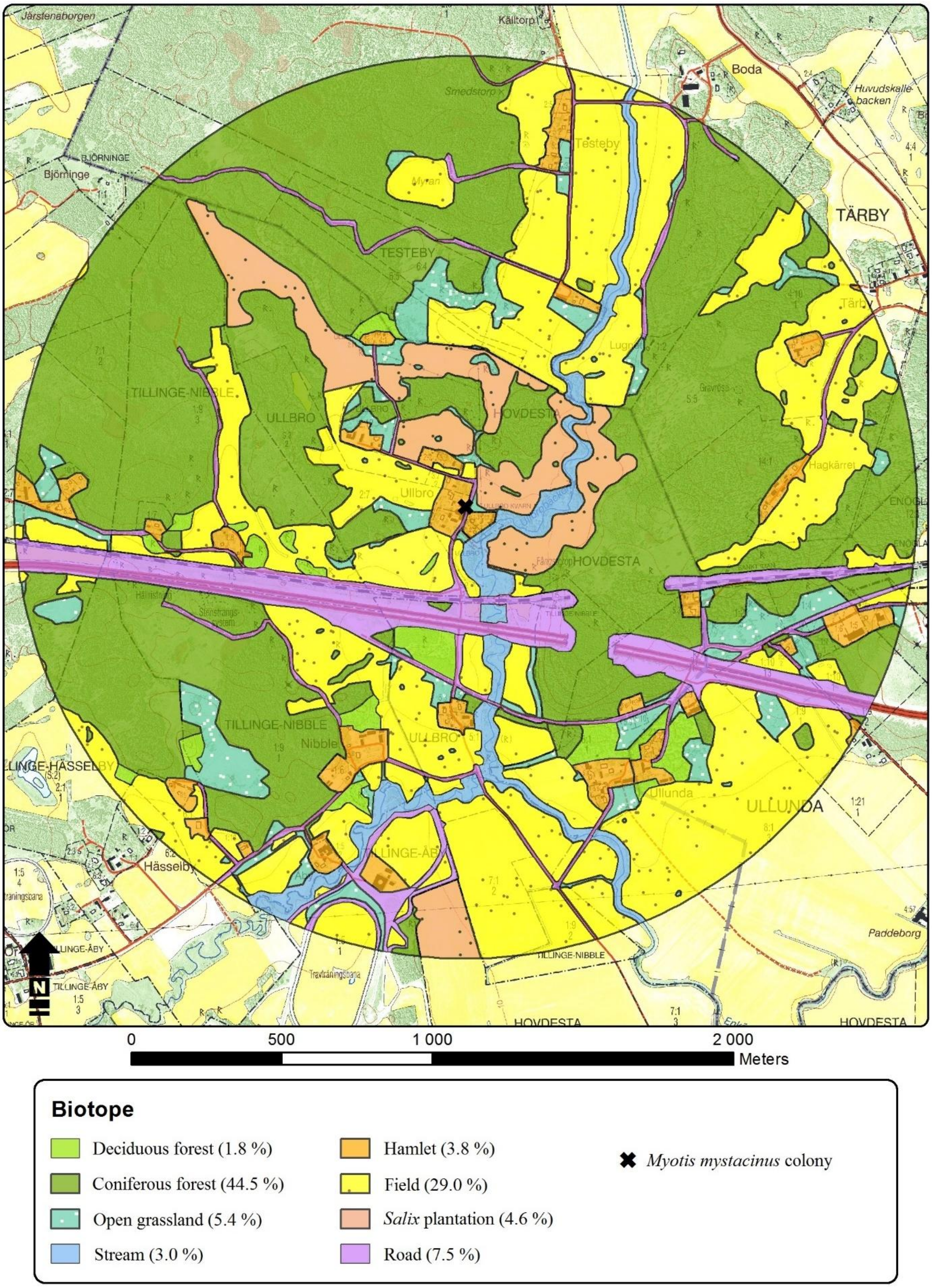

Figure 17 – *Myotis mystacinus* colony and mapped landscape availability of 1.5km radius from the colony

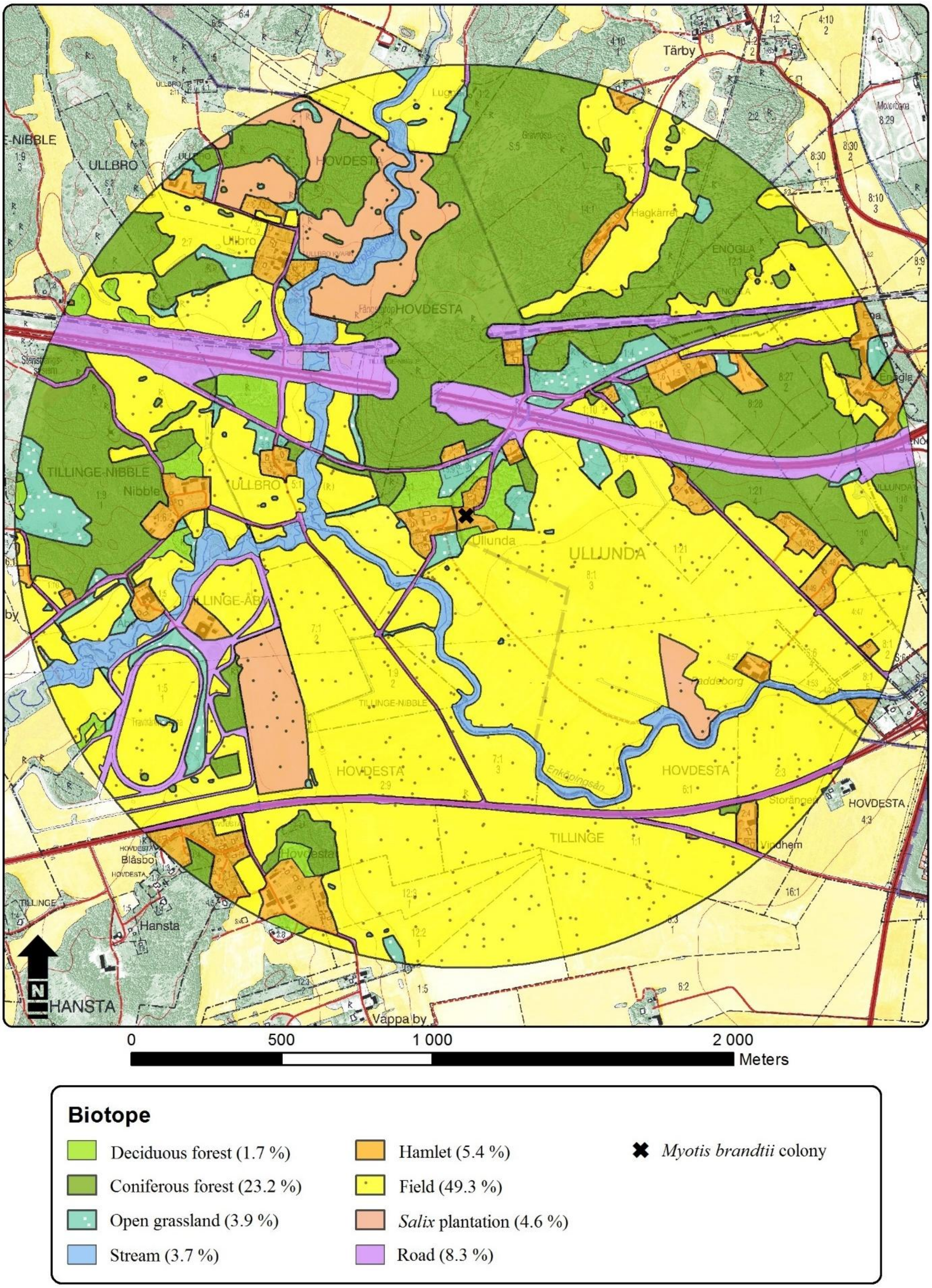

Figure 18 – *Myotis brandtii* colony and mapped landscape availability of 1.5km radius from the colony.

In total, 887 bat positions were taken, 708 of *M. mystacinus* and 179 of *M. brandtii*. Their distribution among biotopes differed from the expected distribution based on biotope availability. In general, bats used more than expected the village, the forest and the river and avoided fields, open grasslands and roads.

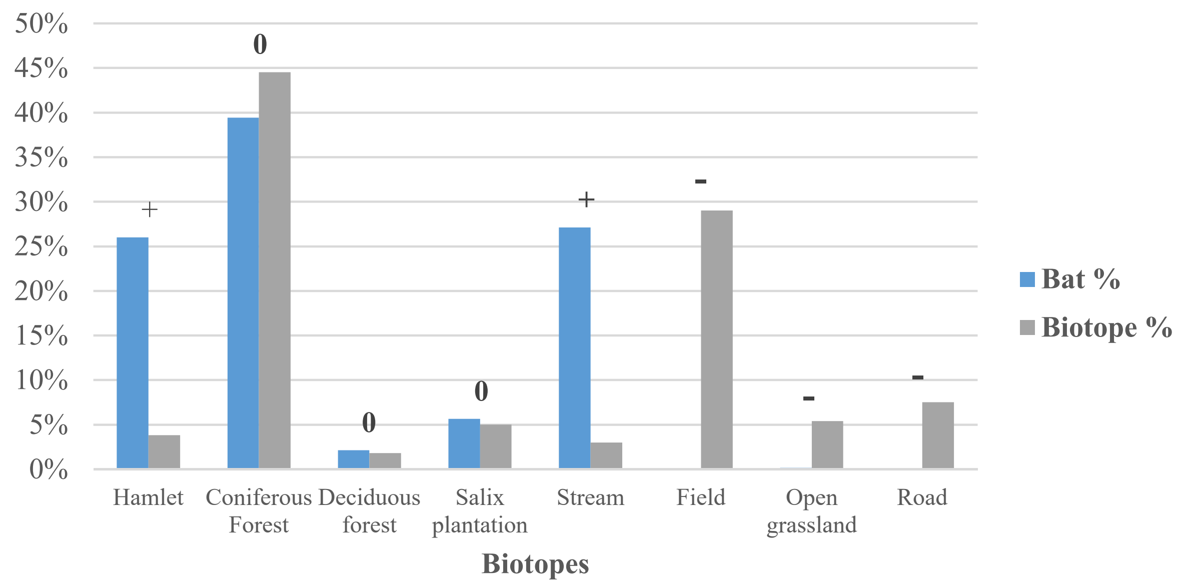

Figure 19 – Bar chart representing the percentage of observations of *M. mystacinus* (blue) and the biotope availability within the landscape (grey) for each biotope. Above each pair of bars, the symbols indicate significance in different usage of biotope compared to expected: plus (+) significantly preferred; minus (-) significantly avoided; and zero (0) no significance.

M. mystacinus (Fig. 19) appears to forage more than expected in the hamlet (26% usage, 4% coverage) and by the stream (27% usage, 3% coverage) compared to its availability. The usage of the coniferous forest (39% usage, 45% coverage), the deciduous forest (2% both usage and coverage) and the *Salix* plantation (6% usage, 5% coverage) was as much as expected. Fields (0% usage, 29% coverage), open grasslands (0% usage, 5% coverage) and roads (0% usage, 8% coverage) were avoided. The followed individuals from this species flew a maximum of 1.3km away from the colony.

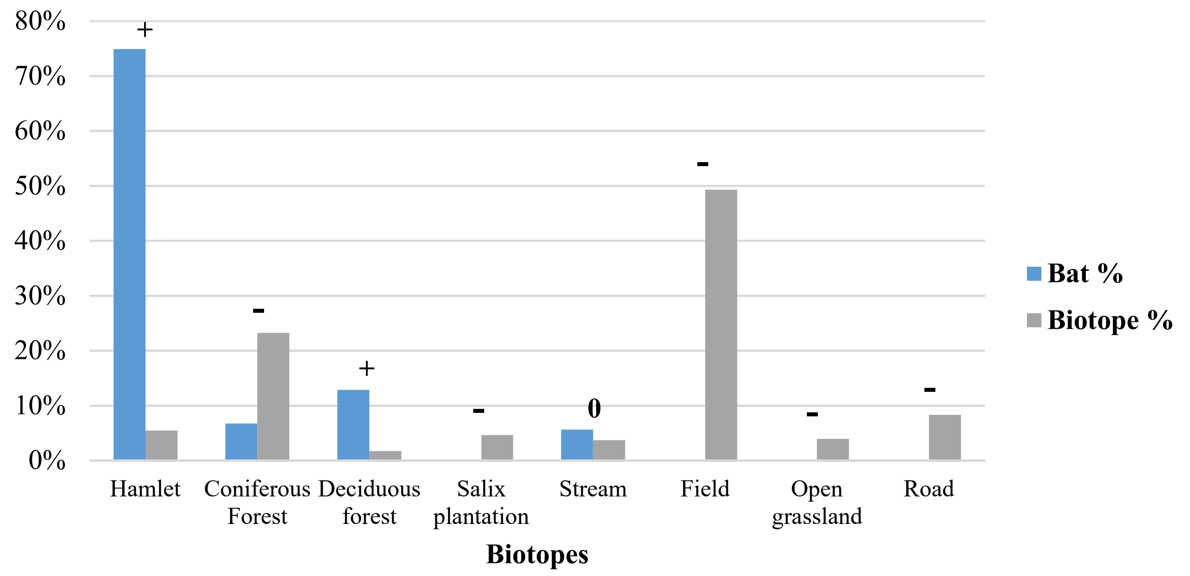

Figure 20 – Bar chart representing the percentage of observations of *M. brandtii* (blue) and the biotope availability within the landscape (grey) for each biotope. Above each pair of bars, the symbols indicate significance in different usage of biotope compared to expected: plus (+) significantly preferred; minus (-) significantly avoided; and zero (0) no significance.

M. brandtii (Fig. 20) reveals a great preference for the hamlet (75% usage, 5% coverage). The deciduous forest (13% usage, 2% coverage) was used more than expected as well. Bats appear to forage in the *Salix* plantation (0% usage, 5% coverage) and the river (6% usage, 4% coverage) as much as expected. Coniferous forest (7% usage, 23% coverage), fields (0% usage, 49% coverage), open grasslands (0% usage, 4% coverage) and roads (0% usage, 8% coverage) were avoided which means, used less than expected. The followed individuals flew a maximum of 500m away from the colony.

No individual crossed the highway or the railway directly. Though, four *M. mystacinus* individuals foraged by the stream close to these infrastructures, whereof two foraged by the stream in the underpass between the highway and railway. No *M. brandtii* individuals approached the highway or railway.

3.2.2. Ultrasound recorders

On the road sites no activity of *Myotis* sp. was registered, while on the stream sites 1482 *Myotis* passes were recorded (Table 4).

Table 4 – Number of *Myotis* sp. passes per ultrasound detector (U. detector sites) placed by the road and the stream. Stream02 and 03 were placed on the underpasses crossing the railway and the highway.

U. detector sites	Period 1	Period 2	Total
Road09	0	0	0
Road10	0	0	0
Stream01	128	7	135
Stream02	274	143	417
Stream03	254	325	579
Stream04	23	14	37
Stream05	5	1	6
Stream06	161	105	266
Stream07	5	37	42
Stream08	1	50	51

Stream02 and 03 were the sites with the highest number of *Myotis* recordings. These sites are both by the under-passage, Stream02 under the railway and Stream03 under the highway. On the other hand, Stream05 had the lower number of *Myotis*, placed by the stream between two fields and near to a small dirt road.

4. DISCUSSION

Globally, these results show that *Myotis mystacinus* and *Myotis brandtii* as expected, avoided the road, and that under- and overpasses were used for foraging and commuting. Moreover, these results demonstrate that even if there was no difference between the usage of gaps and roads, road observations are an overestimation because of the tunnel crossing, indicating that bats avoided roads more than gaps. Activity of *Myotis* species was generally higher in the dark period of the summer season, when compared to the light period. Despite all results, abundance of bats was nearly too low and foraged too often near to their colonies, to draw robust conclusions.

I. How are forest-living species of bats, *M. mystacinus* and *M. brandtii*, affected by roads in a forested dominated landscape in the boreal region?

Habitat selection and avoidance of open spaces:

Both radio-tracking and ultrasound detector survey show that forest areas, stream and hamlet were the main habitats for both species. Open areas, being roads, gaps between forests, fields and open grasslands, appear to be avoided.

Recordings from the ultrasound recorders (3.1.) show that 68% of the observations of *Myotis* species were from the control sites (located in forests) and the wildlife passages (mainly composed by forest) (Table 1). Radio tracking shows that the highest numbers of observations of *M. mystacinus* were in the coniferous forest, stream and hamlet biotopes, though this species used only hamlet and stream areas more than expected when compared to the landscape availability. Regarding *M. brandtii*, the biotopes with highest numbers of observations were hamlet, deciduous forest, which they used more than expected. Data from the ultrasound recorders Stream01 to Stream08 (3.2.2.) revealed high numbers of *Myotis* sp. passes by the stream.

As expected, there is a high number of observations of *Myotis* sp. in forest and stream areas, which might be due to prey availability. The same pattern was found by Lehmann (1984), Vandevelve et al. (2014) and Ciechanowski (2015). The stream was used more than expected by *M. mystacinus*, which is corroborated by Taake (1984) who found this species prefers flowing waters while *M. brandtii* prefers stagnant waters. Although previous studies (Boughey et al., 2011b; Threlfall et al., 2012a; Threlfall et al., 2012b) showed that productive rural areas are highly selected by insectivorous bats, hamlet areas were selected more than anticipated, mainly by *M. brandtii*. This is, however, not surprising since the area is very productive with semi-open habitats composed by some scattered trees and bushes. All three habitats were located close to the colonies, which might explain most of the usage.

Ultrasound recorders data (3.1.1.) revealed that the activity of *Myotis* sp. by the road and in gaps between forests was not significantly different, being in fact very similar according to the very high p-value. However, road observations, even if 22%, were an overestimation because of the tunnel crossing. Tunnels were not responsible for the number of observations in gaps (10%), so it cannot explain the result. This suggests that *Myotis* sp. did not select none of these habitat classes, yet there was more avoidance towards roads than gaps. The tracked bats of both species, *M. mystacinus* and *M. brandtii*, were not found by road, grasslands and field areas. With 0% usage in the three open biotopes, it is evident that there was an avoidance of open areas.

Myotis species appear to avoid these areas, as expected and coherent with previous studies (Vandevelve et al., 2014; Ciechanowski, 2015). Possible explanations for this behaviour include the absence of prey and constrains of spatial orientation (Ciechanowski, 2015), shelter to wind and/or avoidance of predators (Limpens & Kapteyn, 1991), or because they are gaps within the landscape (Bennet and Zurcher, 2013). Possible reasons for the higher avoidance by the road include chemical, light and noise pollution and reduced habitat quality (Coffin, 2007; Benítez-López et al., 2010), though the fact that there was foraging activity just next to the road indicates that the habitat quality is adequate. However, the collected data is rather limited.

The results from the survey revealed a higher activity in every class on the dark period of the summer season, when compared to the light period (3.1.2.). Both forested and open spaces had significantly higher activity on the dark period, except the wildlife passage, which had high activity both periods. It is likely that bats are generally more active and less selective while foraging and commuting in the dark period, due to the higher number of dark hours. If so, bats are in higher danger during this period, since the avoidance of roads is lower.

Road crossing:

None of the followed *M. mystacinus* and *M. brandtii* individuals (3.2.) crossed the road directly, it is likely that they do not risk to fly over it, at least when there are crossing structures as alternative. The ultrasound recorders Road09 and Road10 (3.2.2.) placed by the road but close (around 100-150m) to the wildlife passage showed no *Myotis* sp. passes crossing the road, supporting the radio tracking data. However, some *M. mystacinus* individuals did approach the road and foraged near (distance of tens of meters) to it. These results corroborate those of: Kerth & Melber (2009), who detected significant activity of Bechstein's bat (*Myotis bechsteinii*) on the side of the road but never crossing it; Berthinussen & Altringham (2011) that observed *Myotis* species close to the road, though none of them crossed it; and Vandevelde et al. (2014) identified an avoidance of the edges of a railway by *Myotis* species, when compared to other habitats, noting that this type of linear structure has very weak traffic.

According to the survey result, during the whole summer season (3.1.1.) the difference between the activity of *Myotis* sp. by the road and in the control sites (forest) was nearly significant, with lower observations by the road. However, surprisingly, the results from the direct observations along the road (3.1.5) suggest that no bats crossed the road effectively. Bats appear to use the tunnels to reach the separator between lanes and forage in this vegetation along the road. However, this data does not allow to fully assume that bats do not fly over highways, and it is possible that the few observations at road sites Road01 to Road08 were due to road crossing.

To sum up, *M. mystacinus* and *M. brandtii* were foraging on both sides of the road but in general they did not fly over the road itself. However, bats appear to become less selective while foraging and commuting in the dark period of the summer season, increasing their activity in forested and open areas. Thus, there is considerable evidence to suggest that the road alone is an absolute barrier, and few individuals might cross the road when there are other alternatives.

II. How will crossing structures impact on *M. mystacinus* and *M. brandtii* behaviour?

The highest percentage of *Myotis* sp. passes recorded by the ultrasound recorders (Table 1) was in wildlife passages, with 46% of all observations. Compared with the passes recorded by the road area, the usage of the wildlife passage was considerably higher. Its usage was similarly frequent in both light (3.1.3.) and dark (3.1.4.) periods of the summer season, which implies that this structure is important, regardless of the number of night hours. Radio tracking (3.2.1.) showed that a great part of the positions of *M. mystacinus* and some *M. brandtii* were located in the wildlife passage near to the colony. It is possible that this usage was mainly for foraging instead of crossing. Some *M. mystacinus* individuals were also found foraging by the underpass.

There might be a preference from some *Myotis* sp. to forage by the crossing structures, possibly due to their well conserved / preserved areas. This preference for crossing structures was described in previous studies: Abbott et al. (2012a), Abbott et al. (2012b) and Ward et al. (2015) documents a preference for underpasses. Especially if streams, forests and forest edges continue all the way to the road and through the crossing structure (Ward et al., 2015), as is in the case of the structures considered in this study. On the other hand, Berthinussen & Altringham (2012) reported the ineffectiveness of crossing structures for bats movements.

Data from the direct observations along the road (3.1.5.) show a surprising frequent use of tunnels to reach the separator that divides both lanes. Although Bach et al. (2004) observed that *M. mystacinus* and *M. brandtii* regularly used tunnels to commute along their flight lines, these data suggests that tunnels are used to get to the separator, which is highly selected for foraging.

During the first stage of the fieldwork, colonies, needed for the radio tracking, were searched within the area along the highway. Despite the low number of colonies found, the two encountered colonies were near to the wildlife passage A, which might suggest that bats form their colonies rather near to this structures, where landscape permeability is assured.

Thus, the road worked as a barrier and crossing structures were used for foraging and commuting. Bats were seen using wildlife passages and under passes, but also crossing tunnels, to reach the vegetated separator of the highway to forage along the tree line. These structures appear to be very effective in assuring not only landscape permeability, but are also reasonably good foraging areas.

5. CONCLUSIONS

Surveys show a main usage on forested areas, such as control areas and wildlife passages, and radio tracking shows that both species foraged and commuted on both sides of the road, in forest, stream and hamlet areas.

Regarding avoidance behaviour, radio tracking results showed an avoidance of open areas in general, being fields, open grasslands and the road. Surveys showed few use of gaps in the forest but more usage by the road than expected. However, the direct observations showed that their presence by the road is at least partly explained by the tunnels. Bats crossed the tunnels under the road to reach the separator that divides both lanes and foraged along the separator. This separator is vegetated and might be an area with a relatively high abundance of flying insects.

No bats were seen flying over the road, both during radio tracking and the direct observations however, it is possible that some bat passes by the road were due to road crossing. With this results it is possible to assume that the road is a barrier in the landscape, and that forest-living bats avoid it, more they avoid gaps in the forest. Traffic volume, noise, chemical and light pollution can possibly explain higher avoidance by the road.

As a consequence of higher number of dark hours, *Myotis* sp. increased their activity of both forested and open areas in the dark period of the summer season. Bats appear to be less selective while moving and foraging, being more active even in open areas.

Both surveys and radio tracking data showed a high selection of the wildlife passages for both commuting and foraging. The tracked *M. mystacinus* also selected the underpass near to their colony as hunting ground. Direct observations along the road showed activity by the tunnels connecting both sides of the road and the separator in between. Globally, three types of crossing structures were selected by bats, proving their quality and effectiveness.

Summarising, *Myotis* species select habitats near to the roads, indicating that the habitat quality is adequate. Yet, they appear to avoid open spaces and even more the road itself. With higher number of night hours, might be that bats sometimes take the risk of flying over the road, though. Crossing structures, tunnels and overpasses, have a very important role in *Myotis* populations, used for foraging and commuting. Unexpectedly, crossing structures can also be used to reach the separator in the mid part of the road, so that they forage along this area.

Overall, abundance of bats in this area is too low to draw robust conclusions. Further investigations should last more than one year for more data to be gathered regarding the studied species. The reason why bats select the separator between lanes in the highway requires further investigation. This study focused on *M. mystacinus* and *M. brandtii*, therefore in the future the behaviour of other species occurring in boreal regions near to roads should be investigated. Bat activity belonging to colonies near to roads should also be compared when in the presence of crossings structures and when in absence of these.

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7. SUPPLEMENTARY MATERIAL

Table S 1 – Total number of *Myotis* sp. passes per site by the road, in gaps, in controls and in wildlife passages.

Road sites	Number of observations
Road01	7
Road02	3
Road03	153
Road04	7
Road05	1
Road06	598
Road07	23
Road08	42
Total	834

Gap sites	Number of observations
Gap01	64
Gap02	21
Gap03	30
Gap04	36
Gap05	15
Gap06	6
Gap07	21
Gap08	166
Total	359

Control sites	Number of observations
Control01	79
Control02	26
Control03	8
Control04	41
Control05	182
Control06	66
Control07	288
Control08	85
Control09	107
Control10	7
Total	852

W. Passage sites	Number of observations
WPassage01	157
WPassage02	71
WPassage03	128
WPassage04	257
WPassage05	28
WPassage06	41
WPassage07	324
WPassage08	700
Total	1706

Table S 2 - Radio tracking data: number of nights, number of positions for Myotis mystacinus and Myotis brandtii, number of females and males for each species and number of females in lactation and pregnancy.

Number of nights	37
Number of positions of *M. mystacinus*	708
Number of positions of *M. brandtii*	179
Number of females of *M. mystacinus*	9
Number of males of *M. mystacinus*	2
Number of females of *M. brandtii*	4
Number of males of *M. brandtii*	0
Number of females in lactation	13
Number of females in pregnancy	0

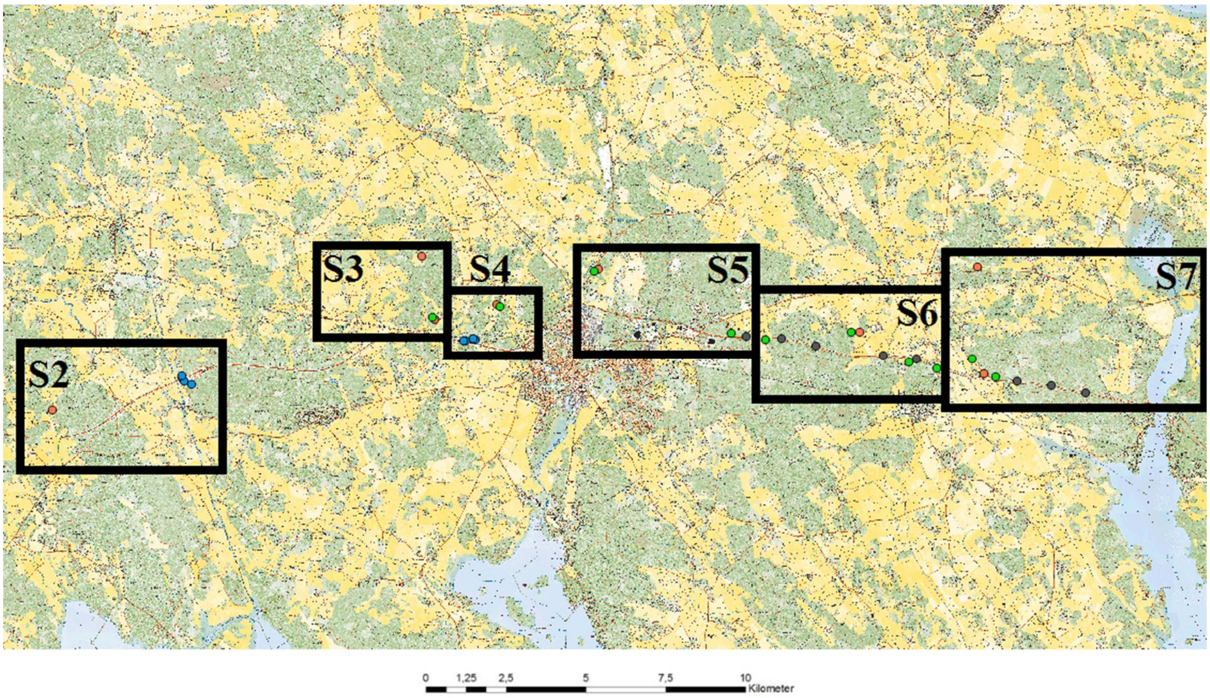

Figure S 1 – Study area: Circles represent the sites where ultrasound recorders were placed: (grey) road sites 1 to 8; (orange) gap sites 1 to 8; (green) control sites 1 to 10; (blue) wildlife passage sites 1 to 8. The boxes represent the following figures.

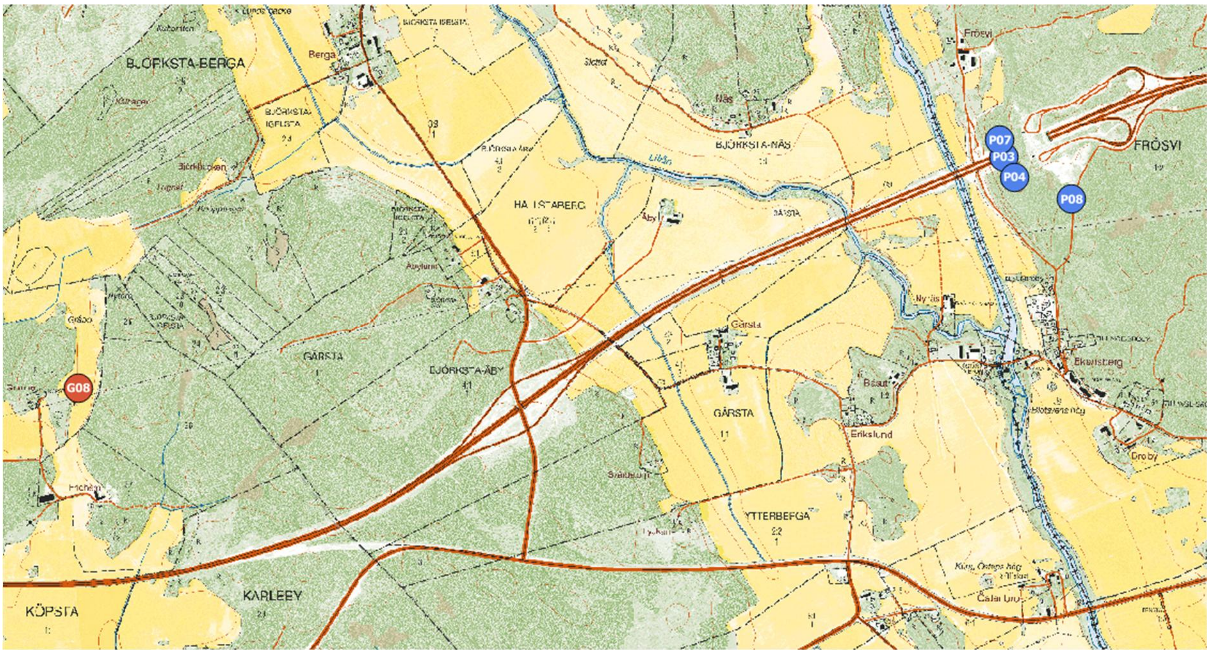

Figure S 2 – Ultrasound recorders sites: (orange) gap site 8; (blue) wildlife passage sites 3, 4, 7 and 8.

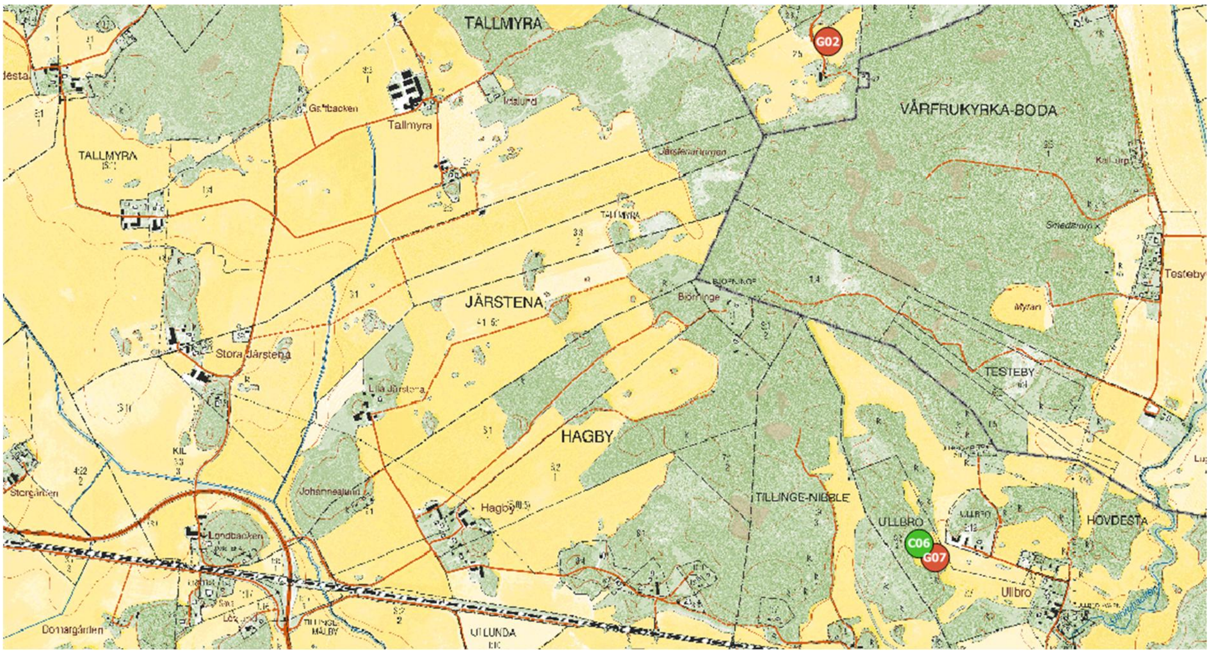

Figure S 3 – Ultrasound recorders sites: (orange) gap sites 2 and 7; (green) control site 6.

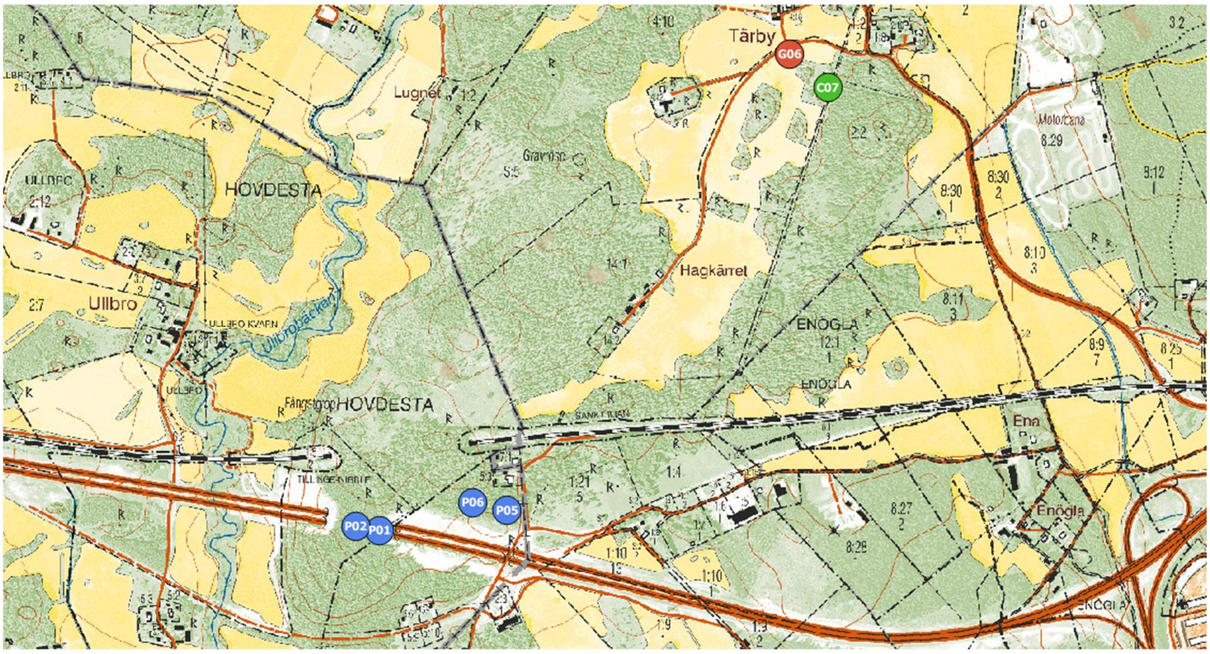

Figure S 4 – Ultrasound recorders sites: (orange) gap site 6; (green) control site 7; (blue) wildlife passage sites 1, 2, 5 and 6.

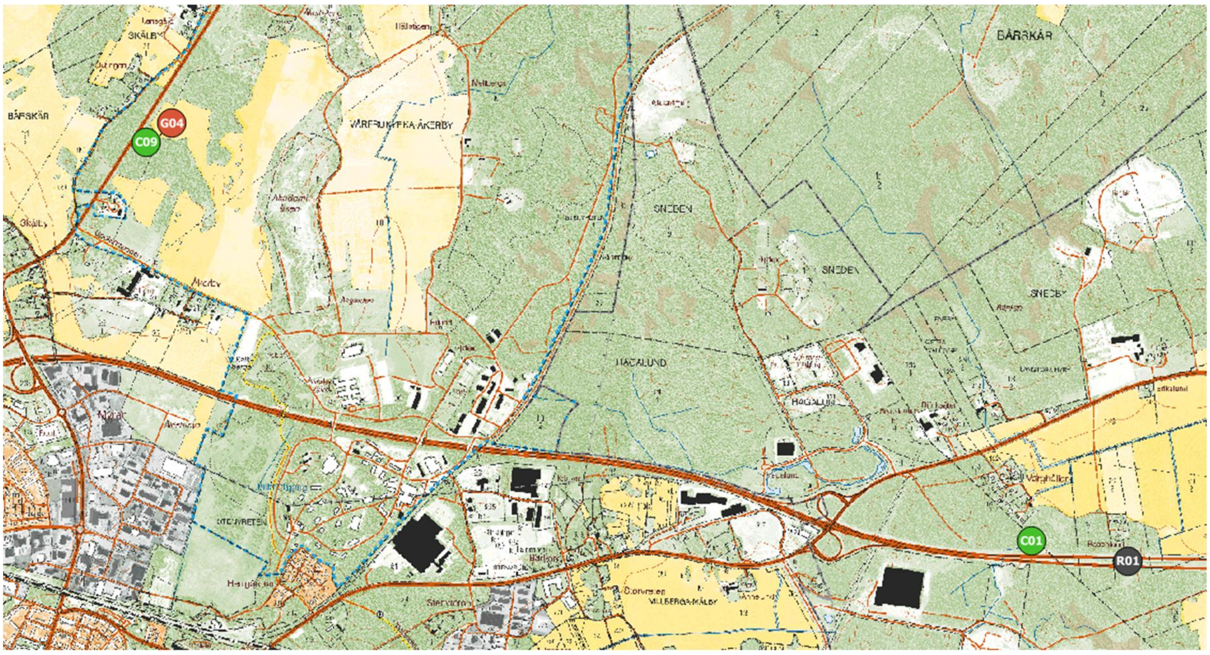

Figure S 5 – Ultrasound recorders sites: (grey) road site 1; (orange) gap site 4; (green) control sites 1 and 9.

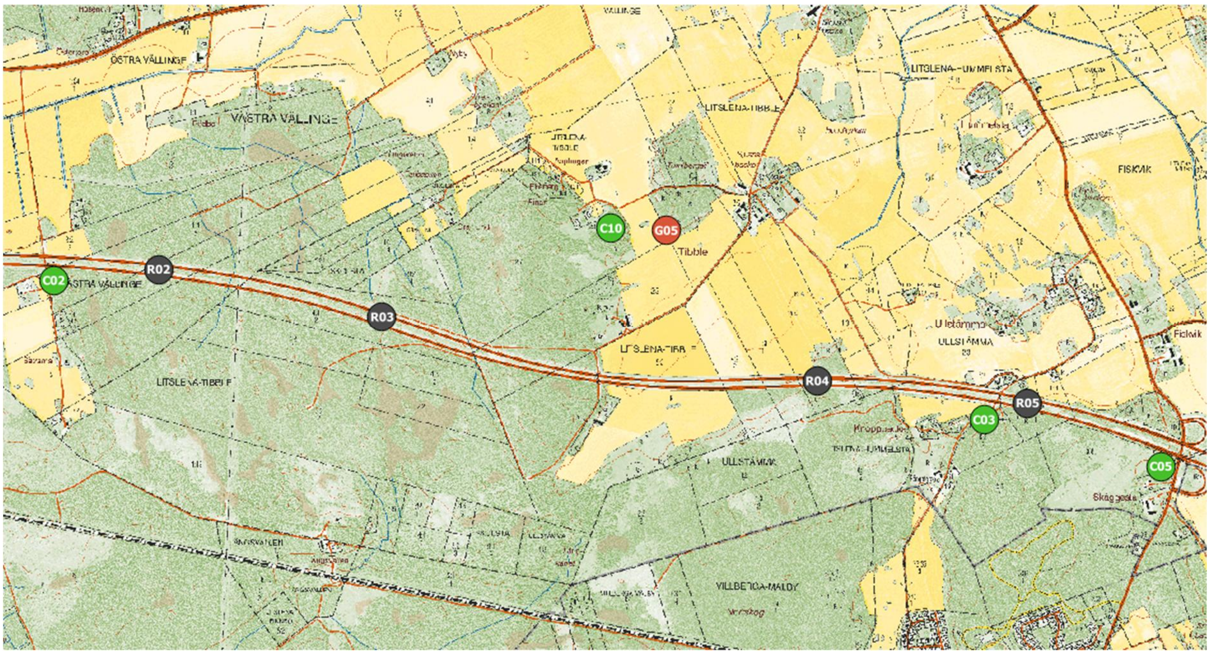

Figure S 6 – Ultrasound recorders sites: (grey) road sites 2 to 5; (orange) gap site 5; (green) control sites 2, 3, 5 and 10.

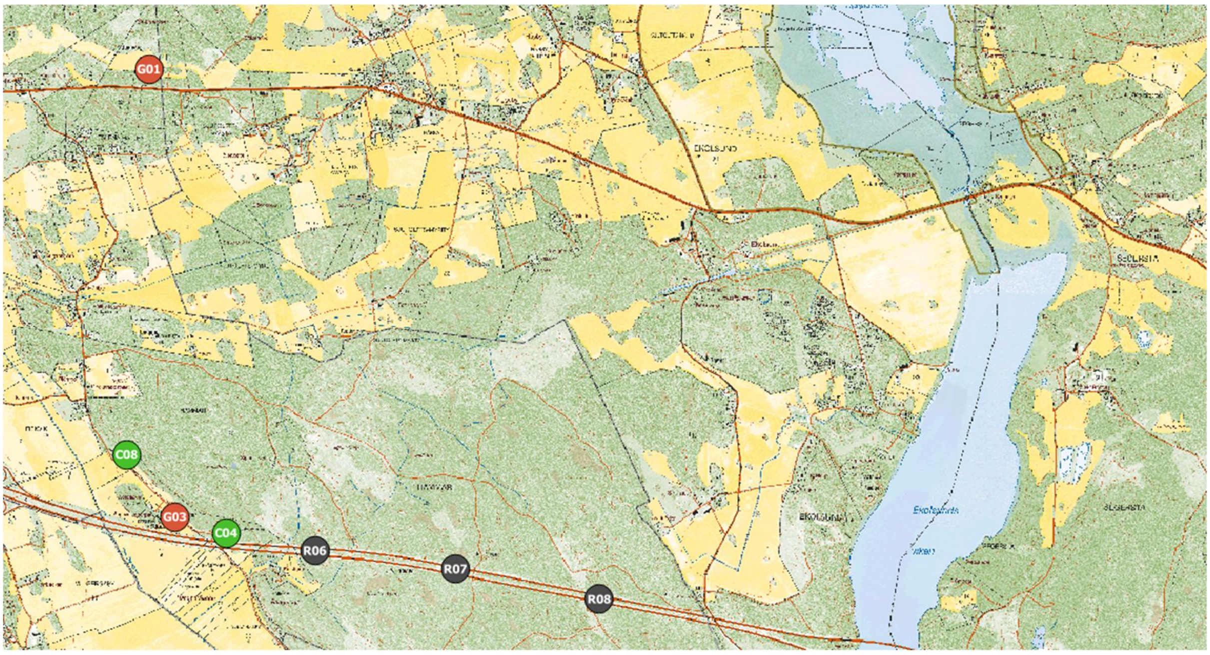

Figure S 7 – Ultrasound recorders sites: (grey) road sites 6 to 8; (orange) gap sites 1 and 3; (green) control sites 4 and 8.